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ORIGINAL ARTICLE

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Antitumoral properties of two new vanadyl(IV) complexes in osteoblasts in culture: role of apoptosis and oxidative stress

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Abstract *Background*: Vanadium derivatives have been reported to display different biological effects, and in particular antineoplastic activity has been demonstrated in both in vivo and in vitro studies. *Purpose:* To study the effect of two new organic vanadyl(IV) complexes (one with glucose, GluVO, and the other with naproxen, NapVO) in osteosarcoma cells. *Methods*: UMR106 osteosarcoma cells and, for comparison, nontransformed MC3T3E1 osteoblasts were used. Proliferation and differentiation were assessed using the crystal violet assay and ALP specific activity, respectively. Morphological alterations were assessed by light microscopy. Lipid peroxidation was evaluated in terms of production of thiobarbituric acid-reactive substances (TBARS) and apoptosis was measured using annexin V. Extracellular regulated kinase (Erk) activation was investigated by Western blotting. *Results*: Vanadium complexes caused morphological alterations and they strongly inhibited UMR106 cell proliferation and differentiation. In contrast, in MC3T3E1 cells, these vanadium derivatives had a relatively weak action. In UMR106 tumoral cells there was a significant increase in TBARS production. Both vanadium complexes induced apoptosis and activation of Erk. PD98059, an inhibitor of Erk phosphorylation, did not block the vanadium-induced antitumoral action. However, the antioxidants vitamins C and E abrogated the apoptosis and TBARS production induced by the vanadium complexes. *Conclusions*: GluVO and NapVO exerted an antitumoral effect in UM106 osteosarcoma cells. They inhibited cell proliferation and differentiation. While the Erk cascade seems not to be directly related to the bioactivity of these vanadium derivatives, the action of both vanadium complexes with organic ligands may be mediated by apoptosis and oxidative stress.

Keywords Vanadium complexes · Anticancer activity
Osteoblasts · Apoptosis · Oxidative stress

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Introduction

Vanadium derivatives have been reported to display different biological effects [6, 7, 11, 12, 13, 29]. In particular, antineoplastic activity has been demonstrated in both in vivo and in vitro studies [1, 3, 8, 9, 11, 19, 23, 26, 28]. Nevertheless, the mechanism involved in the biological effects of vanadium and its derivatives remains unclear. Previous experimental data support the hypothesis that these compounds exert their actions through oxidative stress [7, 40] or the regulation of the cellular levels of tyrosine phosphorylation through either the inhibition of protein tyrosine-phosphatases (PTPases) [21, 25, 32, 33] or the stimulation of tyrosine kinases [34].

We have recently synthesized and characterized two new vanadyl(IV) derivatives, one with naproxen (NapVO) and the other with glucose (GluVO). Preliminary results on the biological properties of these compounds suggest that they could exert antiproliferative effects on osteoblast-like cells in culture [14, 16, 18].

The aim of the present study was to extend our previous observations on the bioactivity of NapVO and GluVO, focusing our attention on their potential anticarcinogenic effects in a rat osteosarcoma-derived cell line (UMR106). Nontumoral MC3T3E1 cells, derived from mouse calvaria, were included for comparison. The signal transduction pathways involved in the antitumorigenic action of these vanadium derivatives were also investigated.

Materials and methods

Vanadium complexes

The complexes of vanadium(IV) with naproxen (NapVO) and glucose (GluVO) were synthesized as previously described [14, 16]. Both ligands were purchased from Sigma Chemical Co. (St. Louis, Mo.) and vanadyl(IV) sulphate was from Merck (Darmstadt, Germany).

Cell culture

MC3T3E1 and UMR106 osteoblast-like cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, Md.) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 95% air and 5% CO_2 as previously described [6, 13, 33, 35]. For experiments, cells were grown in proper culture plates. When the cells reached 80% confluence, the monolayers were washed twice with DMEM and the cells were incubated with NapVO or GluVO at different doses in serum-free DMEM for different periods.

Cell proliferation assay

A mitogenic bioassay was carried out as described by Okajima et al. [31] with some modifications. Briefly, cells in 24-well plates were washed with phosphate-buffered saline (PBS) and fixed with 5% glutaraldehyde/PBS for 10 min. The cells were then stained with 0.5% crystal violet/25% methanol for 10 min. The dye was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 ml/well 0.1 *M* glycine/HCl buffer, pH 3.0/30% methanol, and transferred to test tubes. Absorbance was read at 540 nm. The correlation between cell number and absorbance at 540 nm has been established previously [6].

Cell differentiation assay

Cells were incubated overnight with NapVO, GluVO or the free ligands at different doses in serum-free DMEM. The cell layer was then washed with PBS and solubilized in 0.5 ml 0.1% Triton X-100. Alkaline phosphatase (ALP) activity was measured spectrophotometrically in terms of the initial rates of hydrolysis of *p*-nitrophenylphosphate (p-NPP) to *p*-nitrophenol (p-NP). The production of p-NP was determined by the absorbance at 405 nm. Under these experimental conditions, the product formation was linear for 15 min [6, 27].

Cell morphology

Cells were subcultured in 100-mm dishes and incubated with the vanadium derivatives overnight. At the end of the incubation period, the cells were fixed and stained with Giemsa stain [33]. Cell numbers were determined by counting the stained nuclei in 20 fields per well. For each treatment, 30–100 cells were analysed per field. The mitotic index (MI) was defined as the number of mitotic figures per field over the total number of cells per field [8].

Lipid peroxidation

Lipid peroxidation was measured using a thiobarbituric acid-reactive species (TBARS) assay according the method of Ohkawa et al. [30]. Cell extracts were mixed with 20% acetic acid, pH 3.5, and 0.78% thiobarbituric acid and heated at 95°C for 1 h. The protein content in the cell extract was assessed by the method of Bradford [5]. Lipid peroxide levels were expressed in terms of nanomoles malondialdehyde (MDA) produced per milligram protein, using 1,1,3,3-tetramethoxypropane as standard [7].

Apoptosis assay

The cells were grown on glass coverslips and apoptosis was measured using an annexin V-FITC/propidium iodide (PI) assay (Molecular Probes, Eugene, Ore.) following the recommendations of the manufacturer. The annexin V/PI method is based on the observation that in early stages of apoptosis most cell types translocate phosphatidyl serine from the inner to the outer face of the plasma membrane. This phospholipid can react with annexin V, and the cells will show a green fluorescence on the surface. Cells that have lost membrane integrity (necrotic cells) will have a red fluorescent nucleus stained with PI.

Early apoptotic stages were characterized by annexin V-FITC-positive/propidium iodide-negative (V^+/PI^-) staining while advanced apoptotic (or apoptotic/necrotic) cells were V^+/PI^+ [20, 24, 37]. The cell numbers were determined by counting the stained cells (ranging from 40 to 80 cells per field) in seven fields per coverslip. The apoptotic index was defined as the ratio of V^+/PI^- cells per field to the total cell number per field. Similarly, the necrotic index was defined as the number of V^+/PI^+ cells per field over the total cell number per field.

The effects of vitamins C and E and PD98059 on apoptosis were also tested as described in the section on Erk detection.

Detection of Erk and PErk by Western blotting

Extracellular regulated kinases (Erk) and their activation throughout phosphorylation (PErk) were determined as previously described [17, 18]. The effect of PD98059 (Sigma Chemical Company, St. Louis, Mo.), an inhibitor of Erk kinases, was also assessed. Cells were preincubated with 50 μ*M* PD98059 in DMEM without FBS for 1 h. Then either NapVO or GluVO (1000 μ*M*) was added, and incubation was continued for 1 h at 37°C. Cells were scraped into Laemmli's buffer and the ratio PErk/Erk was determined by Western blotting. PD98059 was prepared in dimethyl sulphoxide (DMSO) and a control with DMSO was also included.

In other series of experiments, the cells were preincubated with vitamin E (50 μ*M*), vitamin C (50 μ*M*) (Sigma) or a mixture of the two (50 μ*M* each) for 6 h at 37°C. Then vanadium complexes were added and the cells were incubated for 1 h at 37°C. The samples were analysed by Western blotting.

Statistical methods

At least three independent experiments were performed for each experimental condition. Results are expressed as the means ± SEM. The significance of differences were determined using Student's *t*-test.

Results

Bioactivity of NapVO and GluVO in osteoblast-like cells

We studied the morphological alterations caused by NapVO and GluVO in osteoblast cell cultures and their influence on cell proliferation, mitotic index and cell differentiation.

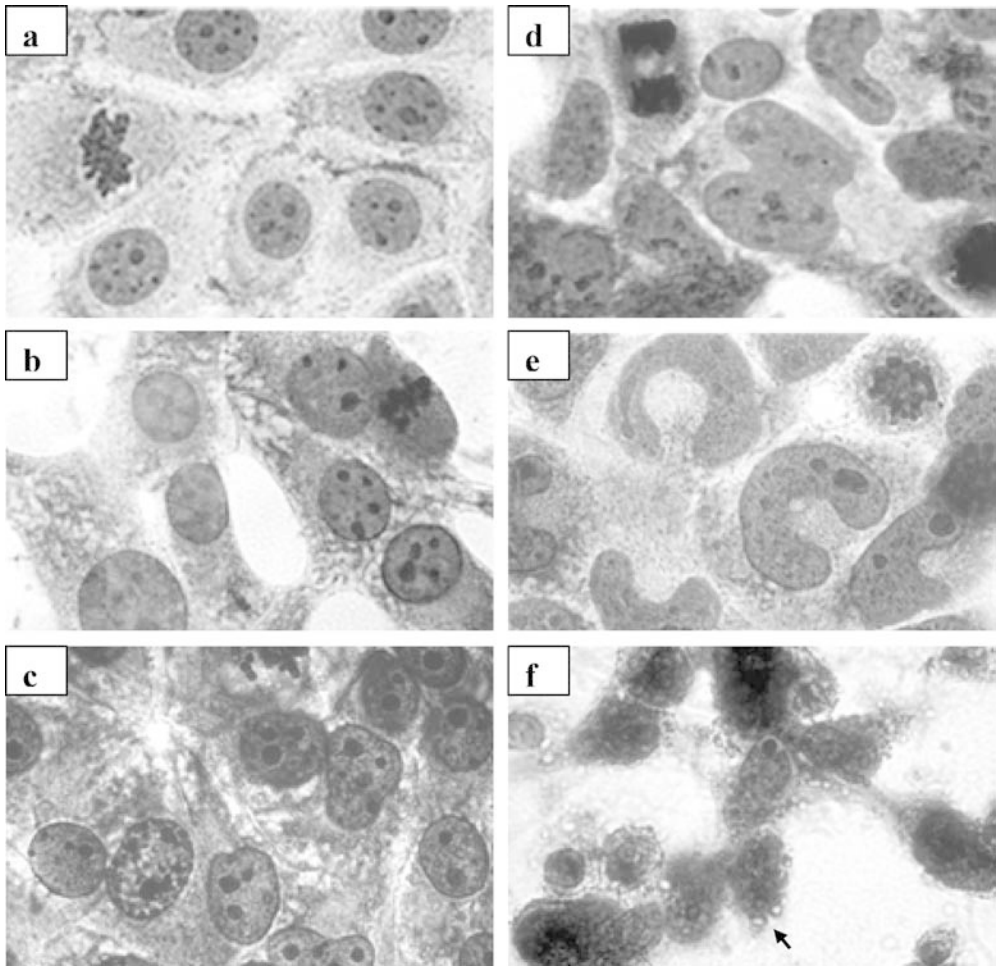

Fig. 1a–f Effect of vanadium compounds on cell morphology. MC3T3E1 (**a–c**) or UMR106 (**d–f**) osteoblasts were incubated in serum-free DMEM (basal control; **a, d**) or serum-free DMEM plus 100 μ*M* NapVO (**b, e**) or 100 μ*M* GluVO (**c, f**) for 24 h. Cells were fixed and stained with Giemsa stain. The *arrow* (**f**) indicates plasma membrane blebs on an apoptotic UMR106 cell after incubation with GluVO (original magnification ×63)

Overnight incubation with vanadium derivatives induced morphological changes in both cell lines. Control MC3T3E1 cells showed the typical features of fibroblast-like cultures (Fig. 1a). Cells exhibited processes connecting them to neighbouring cells. The nuclei were round and contained chromatin granules, and several mitotic figures were also observed. NapVO-treated cells exhibited cytoplasm condensation and vacuolization with a decrease in cellular processes. Nuclei showed condensed chromatin granules (Fig. 1b). In MC3T3E1 cells, GluVO caused stronger effects than NapVO, altering the shape of the nuclei, and inducing fragmentation and condensation of the chromatin (Fig. 1c). Figure 1d shows the characteristic features of UMR106 cells. They had polygonal morphology, well-stained nuclei and cytoplasm, and exhibited cellular processes. Mitotic figures were also present. Nuclear alterations were observed after incubation with NapVO, which induced a kidney-like shape with dense chromatin granules (Fig. 1e). GluVO treatment caused a decrease in cell numbers, and nucleus and cytoplasm condensation, with loss of cellular processes (Fig. 1f). Besides apoptotic figures, membrane blebs (arrows) were observed.

Next we studied the effect of these vanadium derivatives on cell proliferation (Fig. 2a, b). Low concentrations of NapVO exerted a slight but significant mitogenic effect in MC3T3E1 cells (114% of basal level at 2.5 μ*M*), while GluVO only stimulated cell growth at 25 μ*M* (108% of basal level; Fig. 2a). At higher concentrations, both vanadium compounds exerted an antiproliferative effect, this effect being more potent in the tumoral UMR106 cells than in the nontransformed MC3T3E1 osteoblasts. GluVO and NapVO caused approximately 17% inhibition of MC3T3E1 cell proliferation at doses between 50 and 100 μ*M* (Fig. 2a). In UMR106 cells, GluVO was inhibitory at all the tested concentrations (2.5 – 100 μ*M*) and showed a higher potency than similar doses of NapVO (Fig. 2b). Antiproliferative effects of NapVO were detected at doses of 50 to 100 μ*M* (ranging from 10% to 35% inhibition). These vanadium-induced growth inhibition effects were not reversed by vitamins E and C or PD98059 (data not shown).

The effects of both vanadium compounds on the mitotic index in osteoblast cultures were assessed after staining the monolayer with Giemsa stain (Table 1). The mitotic index was significantly decreased in MC3T3E1 cells (30% by 100 μ*M* NapVO and 80% by 100 μ*M* GluVO). In UMR106 cells, 100 μ*M* NapVO decreased the mitotic index by 25%, while 10 μ*M* GluVO

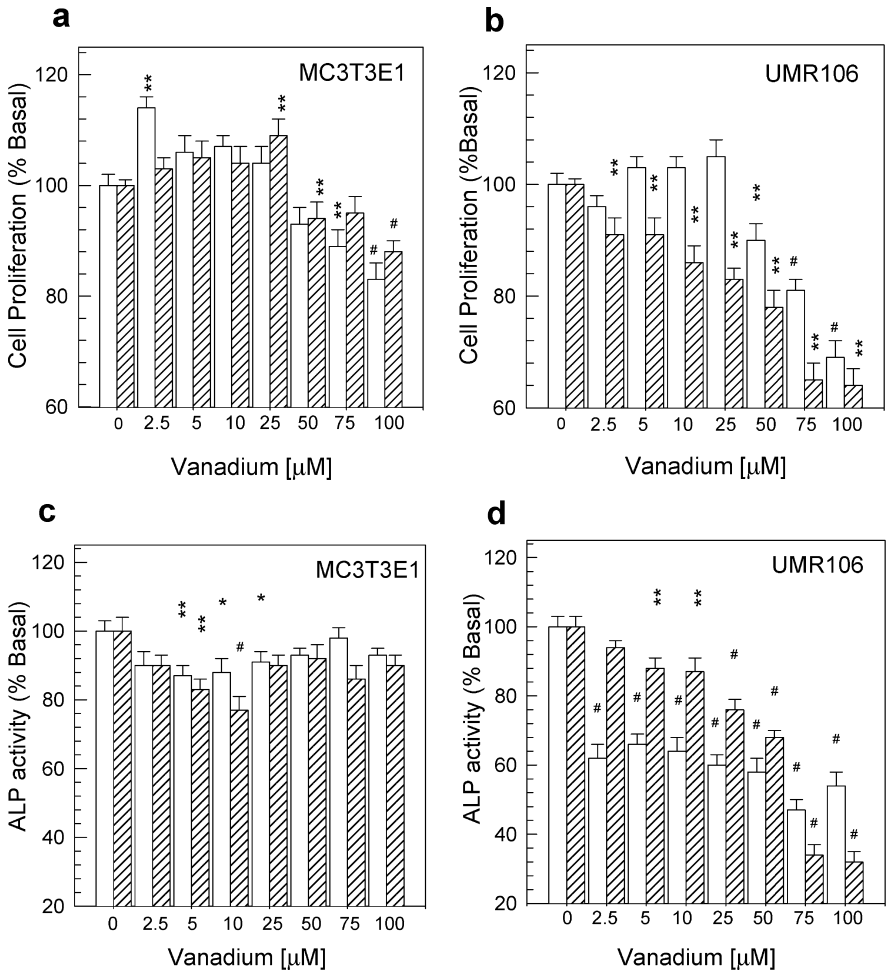

Fig. 2a–d Effect of vanadium compounds on cell proliferation and differentiation. Osteoblasts were incubated in serum-free DMEM alone (*basal*) or with different concentrations of NapVO (*open bars*) or GluVO (*hatched bars*) for 24 h. Cell proliferation (**a, b**) was determined by the crystal violet bioassay. Osteoblastic differentiation (**c, d**) was assessed in terms of ALP specific activity. The results are presented as percentage of basal levels and are means ± SEM ($n = 16$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$, vs basal level

Table 1 Effect of vanadium complexes on the mitotic index of UMR106 and MC3T3E1 cells. Values are means ± SEM ($n = 60$)

Cell line	Vanadium compound	Concentration (μ*M*)	Mitotic index
MC3T3E1	–		$0.030 \pm 3.4 \times 10^{-3}$
	NapVO	10	$0.026 \pm 4.0 \times 10^{-3}$
		100	$0.020 \pm 3.6 \times 10^{-3}$*
	GluVO	10	$0.020 \pm 4.1 \times 10^{-3}$
		100	$0.006 \pm 2.4 \times 10^{-3}$**
UMR106	–		$0.040 \pm 2.6 \times 10^{-3}$
	NapVO	10	$0.050 \pm 4.5 \times 10^{-3}$
		100	$0.030 \pm 4.1 \times 10^{-3}$*
	GluVO	10	$0.008 \pm 2.0 \times 10^{-3}$**
		100	0

$^{*}P < 0.05$, $^{**}P < 0.001$, vs basal level

significantly decreased the mitotic index (by 80%). In addition, 100 μM GluVO, totally blocked the mitogenesis of this cell line. This antimitogenic effect was not observed with the free ligands (data not shown).

The effect of vanadium compounds on the osteoblastic phenotype was evaluated by determining the activity of the specific marker ALP (Fig. 2c, d). In the nontransformed MC3T3E1 cells, both NapVO and GluVO (5–25 μ*M*) induced a weak but significant effect on ALP activity (inhibition ranging from 5% to 20%; Fig. 2c). In contrast, in UMR106 cells, 2.5–50 μ*M* NapVO had a more potent effect (about 40% inhibition) on the ALP specific activity than the same doses of GluVO (10–35% inhibition; Fig. 2d). However, at higher doses (75–100 μ*M*), GluVO induced stronger effects (about 70% inhibition) than NapVO (35–40% inhibition) in UMR106 cells (Fig. 2d).

Study of the putative mechanisms of action of GluVO and NapVO

In an attempt to elucidate the possible mechanisms of action of these vanadium derivatives, we studied their capacity to induce lipid peroxidation, apoptosis and activation of Erk.

The role of lipid peroxidation in the bioactivity of vanadium derivatives was investigated in terms of the generation of TBARS (Fig. 3). This assay requires high vanadium concentrations during a relatively short incubation time (4 h), since these end-products have short half times. GluVO, but not NapVO, caused an increase in TBARS production in a dose-response manner in tumoral UMR106 cells (Fig. 3a). In contrast, neither vanadyl complex affected the levels of TBARS in the nontransformed MC3T3E1 cells (Fig. 3a). Furthermore,

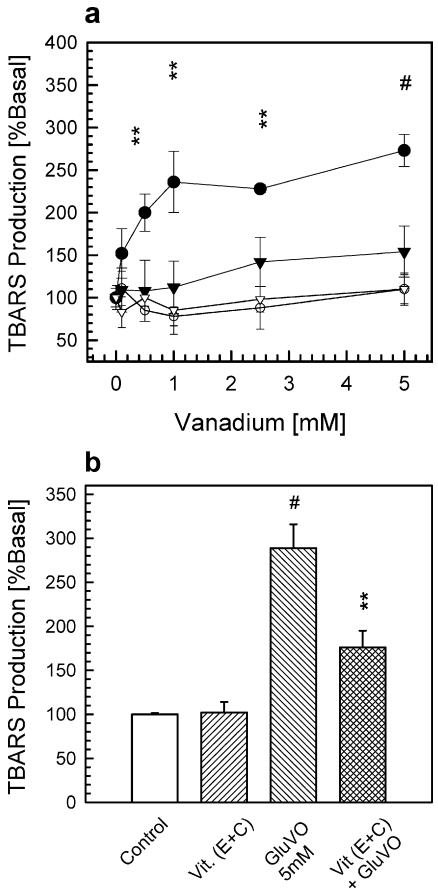

Fig. 3a, b Effect of vanadium derivatives on lipid peroxidation. Osteoblasts were incubated in serum-free DMEM alone (*basal*) or with vanadium compounds for 4 h. Lipid peroxidation was assessed in terms of TBARS production. **a** Dose-response curve for UMR106 (*filled symbols*) or MC3T3E1 (*open symbols*) (*circles* GluVO, *triangles* NapVO). **b** Effect of ROS scavengers (vitamin E and C, 50 μ*M* each) on TBARS production induced by 5 m*M* GluVO. The results are presented as percentage of basal levels and are means ± SEM ($n = 3$). $^{**}P < 0.01$, $^{\#}P < 0.001$, vs basal level

the increase in the levels of TBARS induced by 5 m*M* GluVO was inhibited 40% by a mixture of reactive oxygen species (ROS) scavengers (vitamins E and C) (Fig. 3b).

In a preliminary series of experiments we evaluated the kinetics of the apoptosis induced by 100 μ*M* of either NapVO or GluVO in the two cell lines. In the early apoptotic stages, V^+/PI^- MC3T3E1 cells were detected (48% of the cell population), indicating preservation of the plasma membrane integrity. Membrane blebs were clearly observed (Fig. 4a). During the advanced apoptotic stages, V^+/PI^+ cells were detected (34% of the cell population). Membrane blebs, spikes and well-stained nuclei with nucleoli were detected (Fig. 4b). Similar morphological features were observed in the UMR106 tumoral cells (Fig. 4c, d) (30% of the cell population apoptotic). Apoptosis was observed in both cell lines as early as after 4 h and 2 h of incubation with NapVO or GluVO, respectively. Apoptosis was not detected in cells incubated with the free ligands (naproxen or glucose).

Dose-response studies showed that NapVO and GluVO induced apoptosis in both cell lines in a dose-dependent manner (Fig. 5). NapVO induced the maximal apoptotic response at doses of 100 μ*M* in both cell lines (ranging from 2.3- to 4-fold over basal levels; Fig. 5a, c). No significant alteration in membrane integrity was observed, suggesting that this vanadium compound did not induce necrosis in UMR106 cells. In contrast, in MC3T3E1 cells, 100 μ*M* NapVO caused necrotic processes (5.6-fold over basal levels; Fig. 5a). GluVO produced dose-dependent increases in apoptosis in both cell lines (Fig. 5b, d), the increases ranging from 2.3- to 4.2-fold over basal levels. This compound effectively induced necrosis in UMR106 cells (6-fold over basal levels; Fig. 5d), but had no effect on necrosis in MC3T3E1 cells (Fig. 5b).

Preliminary investigation in UMR106 cells showed that the Erk activation following treatment with the vanadium complexes began following short incubation periods, reaching a maximum after 1 h incubation and remaining at maximal levels for 6 h (data not shown). Both NapVO and GluVO stimulated Erk phosphorylation in a dose-response manner in UMR106 cells (Fig. 6), GluVO more potently than NapVO (400% and 250% over basal levels with 1000 μ*M* GluVO and NapVO, respectively; Fig. 6b and Fig. 6a, respectively). Similar results were observed in MC3T3E1 cells (data not shown).

In order to clarify the signal cascade used by vanadium compounds, the effects of PD98059 and a mixture of antioxidants, vitamins C and E, were investigated. In the UMR106 cells, Erk phosphorylation induced by 1000 μ*M* GluVO was partially inhibited by PD98059 (Fig. 7). Neither vitamin C nor vitamin E alone was able to inhibit vanadium-induced Erk phosphorylation (data not shown), but a mixture of vitamin C and vitamin E partially blocked the activation induced by 1000 μ*M* GluVO. Since vanadium compounds can stimulate ROS production and in turn activate the MAPK cascade, the combined effects of the antioxidants and PD98059 were tested. A mixture of PD98059 with vitamins E and C totally inhibited GluVO-induced Erk activation (Fig. 7).

In order to evaluate the role of oxidative stress and Erk activation on apoptosis caused by these vanadium complexes, we preincubated the cells with a mixture of vitamins C and E (50 μ*M* each) or 10 μ*M* PD98059 (Fig. 8). The mixture of vitamins inhibited NapVO-induced apoptosis/necrosis in UMR106 cells but not in MC3T3E1 cells (Fig. 8a, c). In contrast, the GluVO-induced enhancement of apoptosis/necrosis in the two cell lines was blocked by the vitamin mixture (Fig. 8b, d). PD98059 blocked 30% of the apoptosis induced by NapVO in MC3T3E1 cells but had no effect on apoptosis in UMR106 cells. Furthermore, the necrosis induced by NapVO was totally inhibited by PD98059 in both cell lines (Fig. 8a, c). In contrast, PD98059 blocked 60% of the apoptosis and 100% of the necrosis induced by GluVO in MC3T3E1 cells, but had no effect on the GluVO-induced effects in UMR106 cells. Thus, Erk

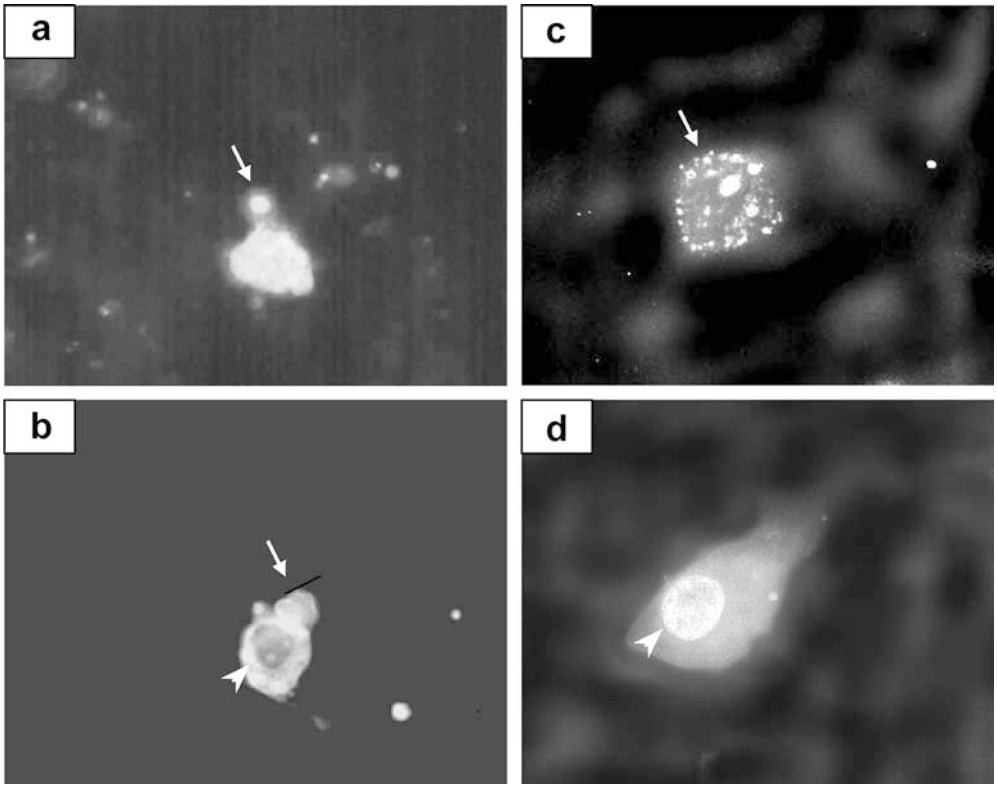

Fig. 4a–d Fluorescence microscopy for apoptosis. Osteoblasts were incubated with 100 μ*M* NapVO for 4 h. Cells were stained with annexin V/PI, fixed with paraformaldehyde and mounted in fluorescence medium. Apoptotic MC3T3E1 cells (**a**) and UMR106 cells (**c**) show plasma membrane stained with annexin V (*arrows* plasma blebs). The figures are representative of the apoptotic population observed per field (48% and 30% apoptosis with 100 μ*M* NapVO in MC3T3E1 and UMR106 cells, respectively). Necrotic MC3T3E1 cells (**b**) (34% of the cell population) and UMR106 cells (**d**) (14% of the cell population) stained with annexin V and PI (*arrow* annexin V-labelled plasma membrane blebs, *arrowhead* nucleus stained with PI)

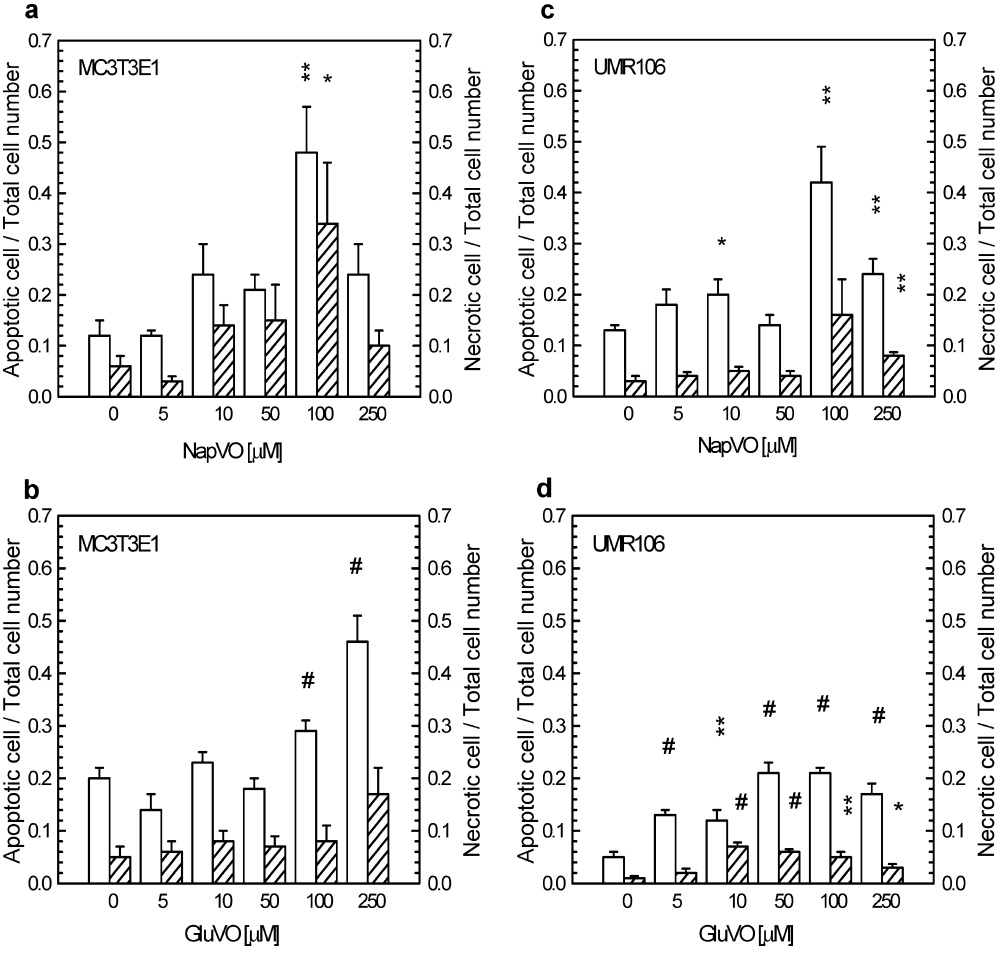

Fig. 5a–d Vanadium-induced apoptosis in osteoblastic cells. Osteoblasts were incubated with different doses of NapVO (**a, c**) for 4 h or GluVO (**b, d**) for 2 h. Cells were stained with annexin V (1 μg/ml) and PI (2.5 μg/ml) for 30 min, fixed with 4% paraformaldehyde for 15 min and mounted in fluorescence mounting medium. The results are presented as apoptotic cell numbers/total cell numbers (*open bars*) or necrotic cell numbers/total cell numbers (*hatched bars*) and are means ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$, vs basal level

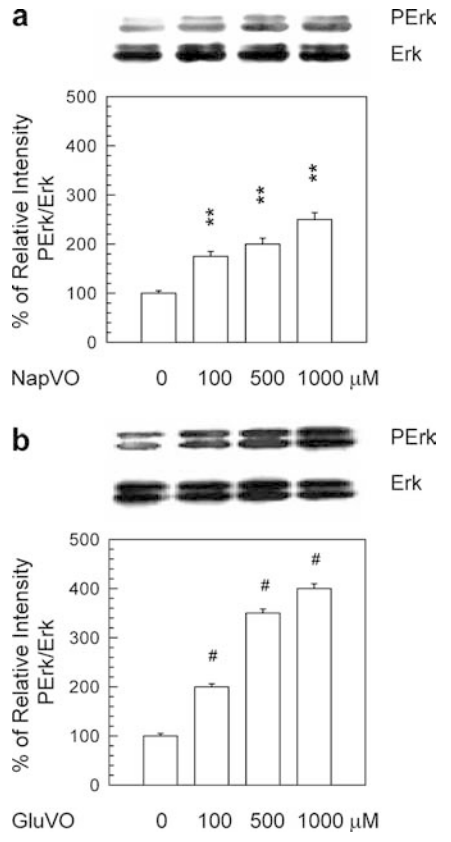

Fig. 6a, b Erk activation by vanadium complexes in UMR106 cells. The effects of different doses of NapVO (**a**) and GluVO (**b**) were evaluated. After incubation with the vanadium compounds (1 h at 37°C), cells were scraped into Laemmli's buffer and analysed by Western blotting using specific antibodies against Erk and PErk. The results are presented as the percentage relative intensity of PErk/Erk and are means ± SEM ($n = 3$). The basal condition (without vanadium) represents 100% Erk activation. $^{**}P < 0.01$, $^{\#}P < 0.001$, vs basal level

activation seems to be involved in the effect of both vanadium compounds in the nontumorigenic MC3T3E1 cells but not in the osteosarcoma UMR106 cells.

Discussion

In recent years there has been increasing interest in the antineoplastic properties of vanadium derivatives [1, 9, 28]. Their efficacy in reducing tumour growth both in vivo and in vitro has been reported [3, 11, 19, 23, 26]. Nevertheless, the mechanism of their action remains unclear.

As part of a research project devoted to developing new vanadium derivatives with potential pharmacological applications, we have synthesized and characterized two novel organic complexes of the vanadyl(IV) cation, one with naproxen and the other with glucose [14, 16]. In the present work we focused on the putative mechanism of action of these compounds. First, we evaluated the effect of GluVO and NapVO on cell morphology, proliferation and differentiation. We observed a biphasic

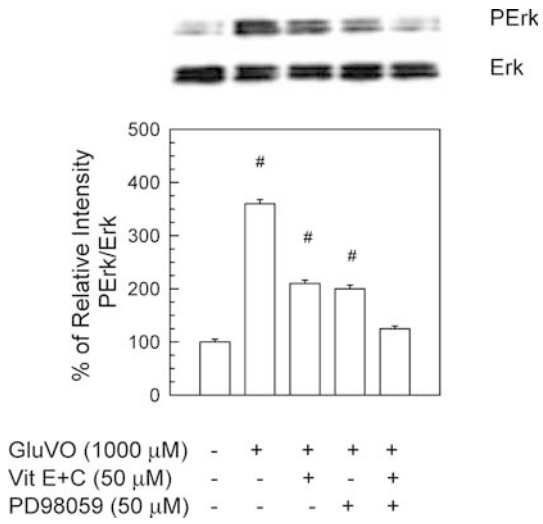

Fig. 7 Effect of Erk activation inhibitors. Cells were preincubated with 50 μ*M* PD98059 (1 h) or a mixture of vitamins E and C (50 μ*M* each, 6 h). After incubation with 1000 μ*M* GluVO for 1 h, Erk was analysed by Western blotting. The results are presented as percentage of relative intensity of PErk/Erk and are means ± SEM ($n = 3$). The basal condition (without vanadium) represents 100% Erk activation. $^{\#}P < 0.001$, vs basal level

effect on MC3T3E1 cell proliferation: both vanadium compounds were stimulatory at low doses (2.5–25 μ*M*) and inhibitory at higher doses (50–100 μ*M*). These findings are in agreement with results previously reported by our group for other vanadium derivatives [13, 15, 16]. On the contrary, an antimitogenic effect was observed in UMR106 cells at all the tested concentrations, with GluVO displaying a stronger inhibitory effect than NapVO. These results are opposite to others previously obtained on the effects of several vanadium compounds on the proliferation of tumoral UMR106 cells, since vanadium derivatives cause in general a stimulatory effect at low doses and have no effect at high concentrations [2, 6, 13, 16]. It is interesting that neither the free vanadyl(IV) cation nor the free ligands induced any antimitogenic effect in tumoral UMR106 cells in the concentration range tested (2.5–100 μ*M*). These observations suggest that the complexation of vanadium(IV) with naproxen or glucose gives it specific and interesting antitumoral properties. These results are in agreement with the changes in the mitotic index obtained in the two cell lines for both vanadium compounds.

Studies on cell differentiation, evaluated in terms of ALP specific activity, showed that both vanadium derivatives had a negative effect on the expression of this phenotypic marker in UMR106 cells, an effect similar to that of other vanadium complexes [2, 6, 13, 15]. The inhibitory effect was almost constant for NapVO (40% inhibition) over the whole range of concentrations, but was dose-dependent for GluVO. In contrast, in MC3T3E1 cells there was a slight inhibitory effect at low doses but no effect at high doses (50–100 μ*M*).

The Erk pathway is classically recognized as a key transducer in the signal cascade mediating cell prolifer-

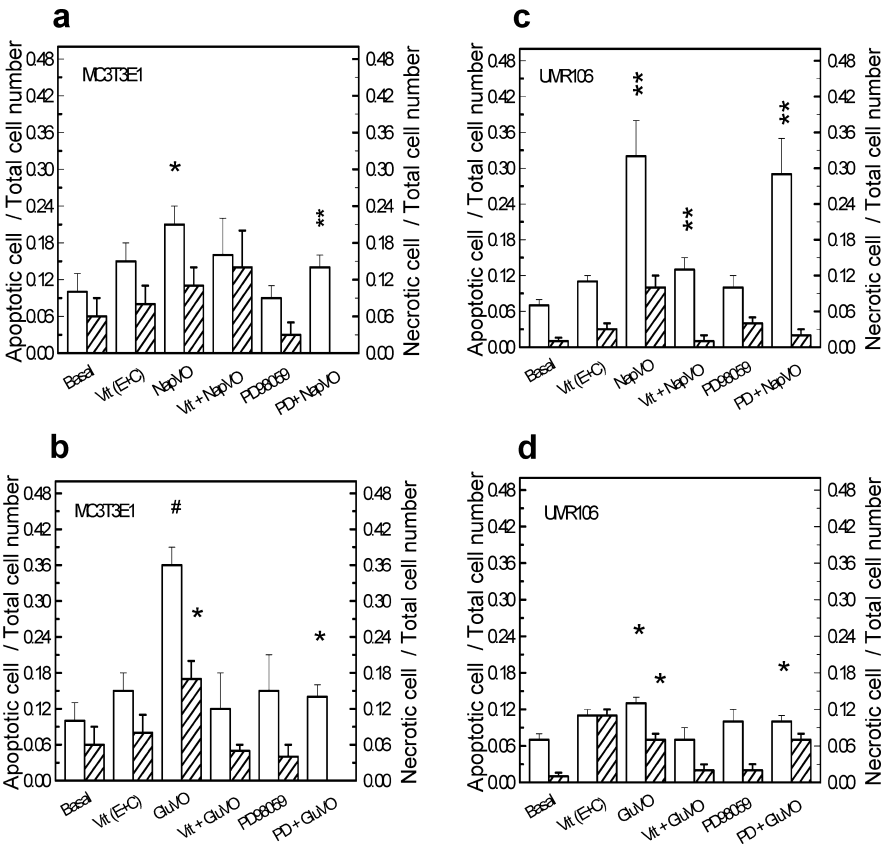

Fig. 8a–d Effect of vitamins and PD98059 on vanadium-induced apoptosis in osteoblasts. After overnight incubation with a mixture of vitamins E and C (50 μ*M* each), MC3T3E1 cells (**a, b**) and UMR106 cells (**c, d**) were incubated with 250 μ*M* NapVO (4 h) or 250 μ*M* GluVO (2 h). Apoptosis was assayed as described in Materials and methods. The results are presented as apoptotic cell numbers/total cell numbers (*open bars*) or necrotic cell numbers/total cell numbers (*hatched bars*) and are means ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$, vs basal level

ation and differentiation [38]. The Erk pathway has also been associated with protection from apoptosis [39]. However, recent investigations have shown that the Erk pathway also mediates cell cycle arrest, antiproliferation, and apoptotic and nonapoptotic death [4]. In an attempt to elucidate the mechanism involved in the effects of NapVO and GluVO, we studied the relationship between Erk pathway activation induced by these vanadium compounds and their effects on cell proliferation and differentiation. As Erk activation is a fast and transient process, we assessed, as others have done [10], the effects of vanadium compounds at 100–1000 μ*M* during 1 h of incubation. A dose-dependent increase in Erk phosphorylation was observed in both cell lines, and this effect was blocked by PD98059 or a mixture of vitamins C and E. An oxidative mechanism seems to be involved in Erk phosphorylation, as others have previously proposed for vanadate [25, 38, 42]. Nevertheless, Erk activation seems not to be related to the effects of either NapVO or GluVO on cell proliferation or on osteoblastic differentiation, since pretreatment with PD098059 did not reverse these effects.

Apoptosis is a physiological process of cell death to provide protection against injuring agents. In some diseases such as cancer, there is an imbalance between the rate of cell proliferation and cell death [22, 40]. In recent years, different metal ions have been reported to be able to induce apoptosis in diverse cell culture models [20, 22]. The morphological techniques used are gold standards for the identification of apoptosis [41]. Apoptotic morphological features observed by microscopy could be used to define this phenomenon, at least qualitatively. The present study showed the ability of NapVO and GluVO to induce apoptosis and necrosis in osteoblastic cell lines. Characteristic apoptotic features, such as membrane blebs and cytoplasm condensation, were observed after treatment with the vanadium derivatives. Quantification of the apoptosis showed that the effect was stronger in tumoral UMR106 cells than in nontransformed MC3T3E1 cells. NapVO also induced necrosis in both cell lines, while GluVO stimulated necrotic processes only in the UMR106 cells. As has been found for other vanadium derivatives, these compounds also stimulate the generation of ROS [7]. Hydroxyl radicals as well as other ROS seem to be apoptosis inducers, probably acting by interacting with the plasma membrane, leading to lipid peroxidation or the activation of cellular apoptotic pathways [36, 40]. To study the effect of ROS production in our model, we analysed the effect of ROS scavengers upon the apoptosis induced by NapVO and GluVO in both cell lines. Our results showed that vitamins C and E partially blocked the apoptotic action of these compounds.

Taken together, our results indicate that apoptosis induced by vanadium complexes may be at least mediated by lipid peroxidation. The antitumoral effect of vanadium compounds in UMR106 osteosarcoma cells seems to be related to oxidative stress but not to Erk activation.

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